AWARD NUMBER: W81XWH-13-1-0027

TITLE: Novel Mechanisms of PARP Inhibitor Resistance in BRCA1-Deficient Breast Cancers

PRINCIPAL INVESTIGATOR: Stephanie Yazinski

CONTRACTING ORGANIZATION: Massachusetts General Hospital, Boston, MA 02114

REPORT DATE: December 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2014	Annual report	1 Dec 2013 - 30 Nov 2014

4. TITLE AND SUBTITLE

Novel Mechanisms of PARP Inhibitor Resistance in BRCA1-Deficient Breast Cancers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0027

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Stephanie Yazinski

E-Mail: SYazinski@mgh.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
55 Fruit Street
Boston, MA 02114-2621

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Mutations in the BRCA1 gene are associated with a heightened lifetime risk for breast cancer. PARP inhibitors (PARPi) have been tested with promising results for the treatment of BRCA1-associated cancers. BRCA1 is essential for error-free repair of DNA double strand breaks via homologous recombination (HR), while PARPs are thought to primarily function in repair of single stranded DNA breaks especially through activation of base excision repair (BER). A synthetic lethal phenotype occurs when BRCA1- deficiency (HR deficiency) is combined with PARPi (BER defect). However, a majority of BRCA1-deficient tumors do not respond to PARPi, and, of those that do, all tumors recur. I aim to (1) To determine how HR is rescued in BRCA1-deficient cells; (2) to elucidate HR-independent mechanisms of PARPi resistance; (3) to elucidate means of targeting PARPi resistant cancers. The results of this project may be broadly applicable to BRCA-like and other non-hereditary, spontaneous breast cancers, including triple negative breast cancers. Ultimately, the proposed experiments will provide an understanding of the mechanisms of acquired resistance to PARPi treatment and how resistant tumors can be categorized and targeted for treatment.

15. SUBJECT TERMS

Breast Cancer, BRCA1, PARP inhibitor, homologous recombination, resistance

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	21	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. Introduction…….4

2. Keywords…….4

3. Overall Project Summary……...4

4. Key Research Accomplishments……8

5. Conclusion……8

6. Publications, Abstracts, and Presentations……..…..…….9

7. Inventions, Patents and Licenses……9

8. Reportable Outcomes..……9

9. Other Achievements……9

10. References……..10

11. Appendices…….11

Introduction:

Mutations in the BRCA1 gene are associated with a heightened lifetime risk for breast cancer (King, Marks, & Mandell, 2003). PARP inhibitors (PARPi) have been tested with promising results for the treatment of BRCA1-associated cancers (Bryant et al., 2005; Gartner, Burger, & Lorusso, n.d.; Tutt et al., 2010). BRCA1 is essential for error-free repair of DNA double strand breaks via homologous recombination (HR) (Gudmundsdottir & Ashworth, 2006), while PARPs are thought to primarily function in repair of single stranded DNA breaks especially through activation of base excision repair (BER) (Krishnakumar & Kraus, 2010). A synthetic lethal phenotype occurs when BRCA1-deficiency (HR deficiency) is combined with PARPi (BER defect) (Bryant et al., 2005)(Farmer et al., 2005)(Edwards et al., 2008)(Helleday, Bryant, & Schultz, 2005). However, a majority of BRCA1-deficient tumors do not respond to PARPi, and, of those that do, all tumors recur. I hypothesize that in order for BRCA1-deficient cells to overcome PARP inhibition, they must acquire mutations or expression changes that alter their DNA damage response, repair pathways, or checkpoint pathways. I further hypothesize that these changes will result in increased sensitivity to other compounds and can be used to develop biomarkers. To this end, I have derived twelve BRCA1-deficient, PARP inhibitor resistant cell lines from the BRCA1-deficent cancer cell line UWB1.289 by two means (Figure 1A and B). I further characterized the homologous recombination (HR) ability of these cell lines as either "HR-restored" or "HR-deficient" based on the level of Rad51 loading, a marker of HR-repair, following DNA damage compared to BRCA1-deficient or BRCA1-rescue cell lines (Figure 2A and B, Figure 3). The objective of this proposal is to determine the how BRCA1-deficient breast cancers become resistance to PARPi and how resistant tumors can be identified and treated.

Key Words: Breast Cancer, BRCA1, PARP inhibitor, homologous recombination, resistance

Overall Project Summary:

Task 1: Determine how HR is restored in BRCA1-deficient cells (Cells with increased Rad51 foci)

1) Test candidate mechanisms of HR restoration:
    a) Determine HR activity in parental, rescue, and PARP inhibitor resistant lines: In my previous annual report, I showed that HR activity in the PARP inhibitor resistant lines varied; however all lines showed at least as much HR activity as was seen in the parental, BRCA1-deficient line using Rad51 and PALB2 as markers.
    b) Test BRCA1-restoration: In my previous annual report, I showed that BRCA1 is not restored in the PARPi resistant lines, and that BRCA1 re-expression does not contribute to PARP inhibitor resistance in these lines.
    c) Test 53BP1 and DNA-PK status: In my previous annual report, I showed that loss of 53BP1, Rif1, or DNA-PK does not contribute to PARP inhibitor resistance in these lines.
2) Identify novel mechanisms of HR restoration:
    a) Confirmation of targets with siRNAs: In my previous annual report, I found that HR activity in BRCA1-deficient cells, both the parental and PARP inhibitor

resistant lines, is dependent on BRCA2 and PALB2, following PARP inhibitor treatment.

b) Confirmation of targets with inhibitors: Because all resistant lines tested were able to load Rad51 to various extents, we can conclude some cell lines were able to partially regain HR function while others were not. Therefore, HR restoration is not required for PARPi resistance. However, all cell lines have some level of HR function, even in the absence of BRCA1, which is PALB2 and BRCA2 dependent (previously shown in annual report). We sought to disrupt the remaining HR in these BRCA1-deficient cells. ATR is a critical regulator of homologous recombination repair; however, the precise role of ATR in HR regulation is unclear. Treatment of U2OS cells with ATR inhibitor (ATRi) but not ATM inhibitor (ATMi) can significantly decrease Rad51 loading following 10Gy IR, confirming that indeed ATR plays a crucial role in HR (Figure 4A and 4B). While ATRi treatment decreases Rad51 loading, there is no effect on BRCA1 loading after IR, suggesting ATR is required for Rad51 loading independently and downstream of BRCA1 (Figure 4C). Furthermore, treatment with ATRi prevented loading of PALB2 in a dose dependent manner following PARP inhibitor treatment in BRCA1-proficient cells (Figure 4D). Since it known that BRCA1, PALB2/BRCA2, and Rad51 load in a sequential manner, this data suggests that ATRi prevents loading of PALB2/BRCA2, which in turn, prevents loading of Rad51, while having no effect on BRCA1 localization after damage. ATRi treatment does not simply delay PALB2 and Rad51 loading, but rather prevents HR repair, as can be seen using the I-SceI reporter assay (Pierce, Johnson, Thompson, & Jasin, 1999). In this assay, an integrated plasmid reporter, DR-GFP, is cut with the rare cutter, I-SceI nuclease, resulting in a double strand break. When this break is repaired via HR, GFP expression occurs, allowing measurement of HR efficiency using flow cytometry. Treatment with increasing doses of either of two ATRi's (VE821 and AZ20) results in significant and dose dependent defects in HR repair, while treatment with a comparable dose of ATM inhibitor (KU55933) has little effect (Figure 4E). All inhibitors prevent activation of downstream targets, as expected (Figure 4F). Taken together, this data suggests that ATR is a critical regulator of HR that functions downstream of BRCA1, but upstream of PALB2 and BRCA2.

I next sought to determine if ATRi could disrupt the HR observed in BRCA1-deficient cells, since ATR acts downstream of BRCA1. It has been previously shown that the defect in HR that results following loss of BRCA1 can be partially rescued by simultaneous loss of 53BP1. U2OS cells were depleted of BRCA1, 53BP1, or both and treated with PARPi to induce damage. Cells were then fixed and stained for Rad51 foci. As expected, following loss of BRCA1, there is a defect in Rad51 loading. This defect is rescued by combined loss of 53BP1, as previously reported. In this BRCA1-deficient context of HR rescue, ATRi significantly inhibits loading of Rad51 (Figure 5A and 5C). Viability assays similarly show loss of BRCA1 alone results in PARPi sensitivity, while combined loss of 53BP1 rescues this sensitivity, but addition of ATRi re-sensitizes the BRCA1 deficient cells to PARPi (Figure 5B and 5C).

I next tested if treatment with ATRi decreased HR in the BRCA1-deficient, but 53BP1-proficient UWB1 cell line and the derived PARPi resistant cell lines. Cells were treated with PARPi or the combination of PARPi and ATRi. Cells treated with the combination of PARPi and ATRi show a decrease in HR relative to cells treated with PARPi alone, using Rad51 as an indicator of HR (Figure 5D and 5E). Furthermore, treatment with ATRi also decreased PALB2 localization to sites of UV laser damage in all BRCA1-deficient cells (Figure 5F and 5G). A laser stripe assay must be used because PALB2 foci are not visible in BRCA1-deficient cells. Taken together, these data demonstrate that ATRi treatment decreases BRCA1-independent HR through disruption of PALB2/BRCA2 loading, thus preventing Rad51 loading.

Task 2: Elucidate HR-independent mechanisms of PARPi resistance (Cells without increased Rad51 Foci)

1) Begin to test candidate HR-independent mechanism of resistance
   a) Test PARP redundancy and efflux pump up-regulation: In my previous annual report, I showed that PARP inhibitor resistance is not due to efflux pump up-regulation or mutations in PARP that prevent PARP inhibitor from binding to its target.

Task 3: Targeting the PARPi resistant breast cancers

To determine the effect of loss of ATR-dependent HR on BRCA1-deficient cells, including PARPi resistant lines, cell viability assays were performed using increasing doses of PARPi in the presence or absence of ATRi after a short treatment period of 5 days (Figure 6A). PARPi alone reduces the viability of only the BRCA1-deficient parental cell line, UWB1, while the rescue line (UWB1+B1) and the resistant lines (SYr) show no effect. ATRi treatment alone has little effect on any cell line. However, the combination of ATRi and PARPi dramatically reduces the viability of all BRCA1-deficient lines, including the PARPi resistant lines. These results were confirmed using a second ATRi (Figure 6B), as well as a Chk1 inhibitor (Figure 6C), as Chk1 is a downstream effector of ATR. While both ATRi's and the Chk1i show a similar synergistic effect when combined with ATRi in BRCA1-deficient cells, a similar dose of ATMi combined with PARPi does not have this effect, suggesting this is specific to inhibition of the ATR pathway (Figure 6D).

A 14 day colony assay revealed the same results, a synergistic decrease in viability specifically in BRCA1-deficient cells following treatment with both PARPi and ATRi (Figure 7A). Furthermore, treatment with increasing doses of ATRi results in a shift of the IC50 to PARPi in the BRCA1 deficient cells, suggesting ATRi is re-sensitizing cells to the PARPi treatment (Figure 7B). The combination has little effect on the rescue cell line exogenously expressing BRCA1. BRCA1-deficient cells, including the PARPi resistant lines SYr12 and SYr13, treated with the combination of ATRi and PARPi do not simply arrest growth, but rather undergo apoptotic cell death, as measured by Annexin V staining, while the rescue cell line expressing BRCA1 is resistant to this combination (Figure 7C).

I next sought to determine if these findings could be extending to other BRCA1-deficient cancer types, including breast cancer. Rad51 foci were stained following

treatment with PARPi or PARPi and ATRi in human breast cancer, BRCA1-deficient HCC1937 cells or HCCwt, an isogenic cell line which exogenously expresses BRCA1. As expected, BRCA1-deficient HCC1937 cells have a partial defect in Rad51 loading following PARPi treatment; however, HCC1937 cells have an even greater defect in Rad51 loading following the combination of PARPi and ATRi treatment (Figure 8A). I next tested the effect of combined PARPi and ATRi treatment in viability assay using HCC1937 cells and HCCwt cells. Treatment with PARPi alone has a little effect in HCC1937 cells, despite a well-established HR deficiency in these cells, suggesting this cancer cell line is PARPi resistant. However, combining treatment with the ATRi re-sensitizes these cells to PARPi, with ATRi decreasing the IC50 of PARPi in a dose dependent manner. The combination had less effect on the isogenic HCCwt cells in which wtBRCA1 is exogenously expressed, as the IC50 of PARPi remained high (Figure 8B and 8C). We further extended these studies to another cell line, BR5, a BRCA1-deficient mouse ovarian cancer cell line which is exceptionally sensitive to PARPi treatment (Xing & Orsulic, 2006). Though BR5 cells are already sensitive to PARPi treatment, these cells become even more sensitive to PARPi treatment, and undergo increased cell death following addition of ATRi treatment (Figure 8D and 8E). Finally, a PARPi resistant line derived from BR5 lines, BR5-R1, shows similar synthetic lethality when PARPi is combined with ATRi, again demonstrating this effect is not cell line specific (Figure 8E and 8F). These results confirm that a variety of BRCA1-deficient cell lines, which are both resistant and sensitive to PARPi treatment, become more sensitive to PARPi in the presence of ATRi.

Finally, I sought to determine if this synthetic lethality seen with combined PARPi and ATRi could be used to target the parental cells to prevent resistance from immerging. To this end, I treated sparsely plated parental UWB1 or rescue UWB1+B1 cells with PARPi alone, ATRi alone, or the combination for 45 days, changing the media and adding fresh inhibitors every three days. While resistant colonies readily immerged from the ATRi treatment alone and a few resistant colonies immerged from the PARPi treatment alone in the UWB1 cells, no colonies were able to survive the prolonged combined PARPi and ATRi treatment (Figure 9). Cells were able to survive and proliferate in the rescue UWB1+B1 cells in all three treatments. Importantly, combining PARPi with the same dose of ATMi did not have this same effect, demonstrating that the combined effect is ATRi specific. Taken together, these results suggest combined PARPi and ATRi treatment selectively targets BRCA1-deficient cells to cell death and prevents PARPi resistance from emerging.

Year 2 Training plan:

- I have been meeting with my mentor bi-weekly and meeting with my co-mentor monthly to discuss results and future experiments.
- I have been presenting at and attending lab meeting, joint journal club, and departmental seminar.

- I was a co-author on three manuscripts with collaborators for use of a technique, the DNA fiber assay, which I developed in the lab to study replication defects in the BRCA1-deficient cells
- I have attended MGH Grand Rounds of several talks related to breast cancer.
- I supervised a rotating graduate student in Harvard's graduate program.

Key Research Accomplishments:

- ATR is a critical regulator of HR
- ATR functions to promote HR down stream of BRCA1 but upstream of PALB2 and BRCA2
- Treatment with ATR inhibitor can disrupt the remaining BRCA1-independent HR remaining in the BRCA1-deficient parental cells as well as the BRCA1-deficient, PARP inhibitor resistant lines.
- Treatment with ATRi re-sensitizes BRCA1-resistant cells to PARPi
- Combination of ATRi with PARPi specifically reduces the viability of BRCA1-deficient cells
- Combination of ATRi with PARPi specifically leads to cell death in the BRCA1-deficient cells
- The combination of ATRi with PARPi specifically reduces the viability of BRCA1-deficient cells in a number of cell lines, including human breast cancer cells, HCC1937, and mouse ovarian cancer cells, BR5-AKT.
- ATRi prevents PARPi resistant colonies from emerging
- I developed new PARPi resistant cells from a mouse BRCA1-deficient ovarian cancer cell line
- Combined ATRi and PARPi can selectively reduce the viability of these PARPi resistant mouse ovarian cells

Conclusion:

In year one of my proposal, I had derived BRCA1-deficient, PARP inhibitor resistant lines from a parental PARP inhibitor sensitive line. I had determined that homologous recombination (HR), as marked by Rad51 loading and PALB2 localization to DNA damage, occurs in all BRCA1-deficient cell lines, including the parental line, and is further restored in several PARP inhibitor resistant lines. I ruled out known mechanisms of PARPi resistance in the absence of BRCA1, including restoration of BRCA1, loss of 53BP1 or Rif1, loss of DNA-PK activity, increased efflux of PARPi from the cell, increased PARP expression, or mutations in PARP that prevent the inhibitor from binding. I used targeted siRNAs in a candidate approach to determine that the restored HR levels, as well as the low level or residual HR seen in the BRCA1-deficient parental cell line depends on BRCA2, PALB2, and MRG15.

In this second year of my proposed experiments, using inhibitors of ATR, I have determined that ATR plays a critical role in HR repair downstream and independently of BRCA1, but upstream of PALB2 and BRCA2. I have determined that ATR plays a role in promoting HR repair even in the absence of BRCA1. Treatment with ATRi prevents HR in the absence of BRCA1, leading to resensitization of these cells to PARPi treatment, resulting in reduced viability specifically in BRCA1-deficient cells, including

PARPi resistant lines. The combination of ATRi and PARPi results in cell death specifically in BRCA1-deficient cells. These findings were extended beyond the BRCA1-deficient UWB1 cells, to a human breast cancer cell line, HCC1937, which is already PARPi resistant through an unknown mechanism, and a BRCA1-deficient mouse ovarian cancer cell line BR5-AKT, which is highly PARPi sensitive. Furthermore, I developed a new PARPi resistant cell line from the BRCA1-deficient mouse BR5-FVB parental line and have found this resistant line is also sensitive to combination of PARPi and ATRi. Finally, using a long term (42 day) colony assay, I have determined that the combination of PARPi and ATRi in a PARPi sensitive cell line can prevent PARPi resistance from emerging. Taken together, the results of the experiments contained in this annual report demonstrate that treatment with ATRi in BRCA1-deficient cancer cells, both ovarian and breast, results in further depletion of HR repair and ultimately, when combined with PARPi, results in cell death. Furthmore the combination ATRi and PARPi prevents PARPi resistant clones from emerging. This suggests that the combination therapy of ATRi and PARPi may be a useful initial therapy as well as a useful treatment for resistant tumors.

Taken together, the results of this years progress on proposed work suggest combined PARPi and ATRi treatment selectively targets BRCA1-deficient cells to cell death and prevents PARPi resistance from emerging. Ultimately, the proposed experiments for year three will provide insight into how ATR functions to restore HR in the absence of BRCA1, how BRCA1-mutated breast cancers acquire resistance to PARP inhibitor treatment, and ultimately how recurrent tumors can be treated.

Publications, Abstracts and Presentations:

Papers published:
The BRCA1-interacting protein Abraxas is required for genomic stability and tumor suppression. Castillo A, Paul A, Sun B, Huang TH, Wang Y, Yazinski SA, Tyler J, Li L, You MJ, Zou L, Yao J, Wang B. Cell Reports. 2014 Aug 7;8(3):807-17. PMID: 25066119

Suppression of genome instability in pRB-deficient cells by enhancement of chromosome cohesion. Manning AL, Yazinski SA, Nicolay B, Bryll A, Zou L, Dyson NJ. Molecular Cell. 2014 Mar 20;53(6):993-1004. PMID: 24613344

PRP19 transforms into a sensor of RPA-ssDNA after DNA damage and drives ATR activation via a ubiquitin-mediated circuitry. Maréchal A, Li JM, Ji XY, Wu CS, Yazinski SA, Nguyen HD, Liu S, Jiménez AE, Jin J, Zou L Molecular Cell. 2014 Jan 23;53(2):235-46. PMID: 24332808

Inventions, Patents, and Licenses: Nothing to report

Reportable outcomes: Nothing to report

Other Achievements: A new cell line, BR5-R1, was derived. This is a PARP inhibitor resistant line that is derived from the mouse ovarian cancer cell line BR5 (Xing & Orsulic, 2006), which is BRCA1-deficient and PARP inhibitor sensitive.

References:

Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., … Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*, *434*(7035), 913–7. doi:10.1038/nature03443

Edwards, S. L., Brough, R., Lord, C. J., Natrajan, R., Vatcheva, R., Levine, D. A., … Ashworth, A. (2008). Resistance to therapy caused by intragenic deletion in BRCA2. *Nature*, *451*(7182), 1111–5. doi:10.1038/nature06548

Farmer, H., McCabe, N., Lord, C. J., Tutt, A. N. J., Johnson, D. a, Richardson, T. B., … Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*, *434*(7035), 917–21. doi:10.1038/nature03445

Gartner, E. M., Burger, A. M., & Lorusso, P. M. (n.d.). Poly(adp-ribose) polymerase inhibitors: a novel drug class with a promising future. *Cancer Journal (Sudbury, Mass.)*, *16*(2), 83–90. doi:10.1097/PPO.0b013e3181d78223

Gudmundsdottir, K., & Ashworth, A. (2006). The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*, *25*(43), 5864–74. doi:10.1038/sj.onc.1209874

Helleday, T., Bryant, H. E., & Schultz, N. (2005). Poly(ADP-ribose) polymerase (PARP-1) in homologous recombination and as a target for cancer therapy. *Cell Cycle (Georgetown, Tex.)*, *4*(9), 1176–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16123586

King, M.-C., Marks, J. H., & Mandell, J. B. (2003). Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science (New York, N.Y.)*, *302*(5645), 643–6. doi:10.1126/science.1088759

Krishnakumar, R., & Kraus, W. L. (2010). The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. *Molecular Cell*, *39*(1), 8–24. doi:10.1016/j.molcel.2010.06.017

Pierce, A. J., Johnson, R. D., Thompson, L. H., & Jasin, M. (1999). XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes & Development*, *13*(20), 2633–2638. doi:10.1101/gad.13.20.2633

Tutt, A., Robson, M., Garber, J. E., Domchek, S. M., Audeh, M. W., Weitzel, J. N., … Carmichael, J. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in

patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. *Lancet*, *376*(9737), 235–44. doi:10.1016/S0140-6736(10)60892-6

Xing, D., & Orsulic, S. (2006). A mouse model for the molecular characterization of brca1-associated ovarian carcinoma. *Cancer Research*, *66*(18), 8949–53. doi:10.1158/0008-5472.CAN-06-1495

Appendices:

Supporting data:

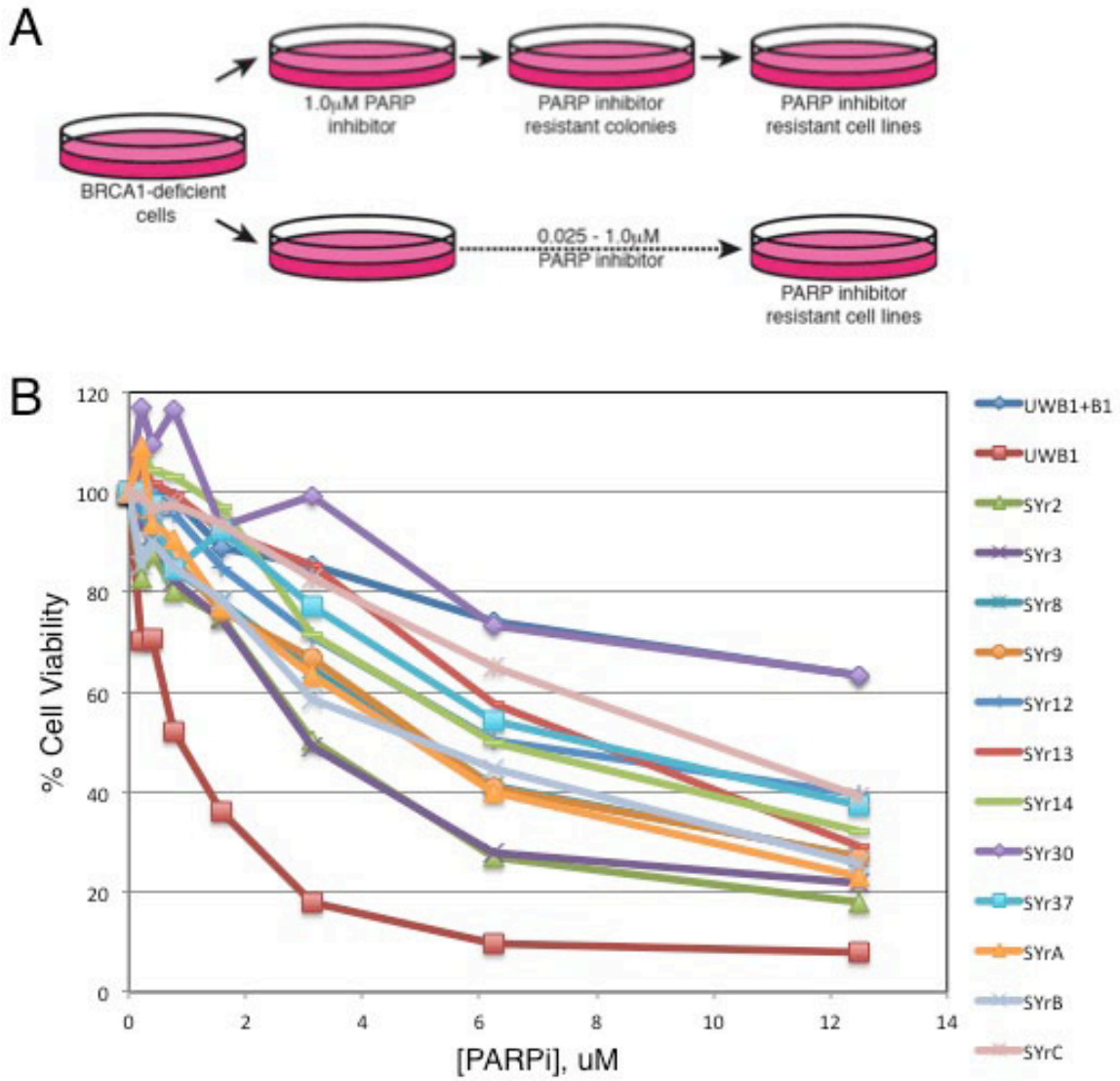

Figure 1: Twelve PARP inhibitor (PARPi) resistant cells lines were derived from a parental, BRCA1-deficient cell line. (A) Schematic of two methods used to derive PARP inhibitor resistant cell lines. In one method (top), parental cells were treated with a high dose of PARPi (1.0uM) such that most cells died. A few surviving cells grew to form resistant colonies after 45 days of treatment. These clones were selected and developed

into nine resistant cell lines. In a second method (bottom), parental cells were treated with a sublethal dose of PARPi (0.025uM) and gradually increased after several passages to 1.0uM to allow cells to gradually adapt to the PARPi treatment. (B) Cell viability curve using CellTiter-Glo viability assay with increasing doses of PARPi. The BRCA1-deficient UWB1 cell line is most sensitive to PARPi, while the isogenic BRCA1-rescue line, UWB1+B1, is resistant to PARPi, demonstrating the sensitivity is due to BRCA1 status. The derived BRCA1-deficient, PARPi resistant lines (SYr) are all more resistant to PARPi than the parental line from which they are derived.

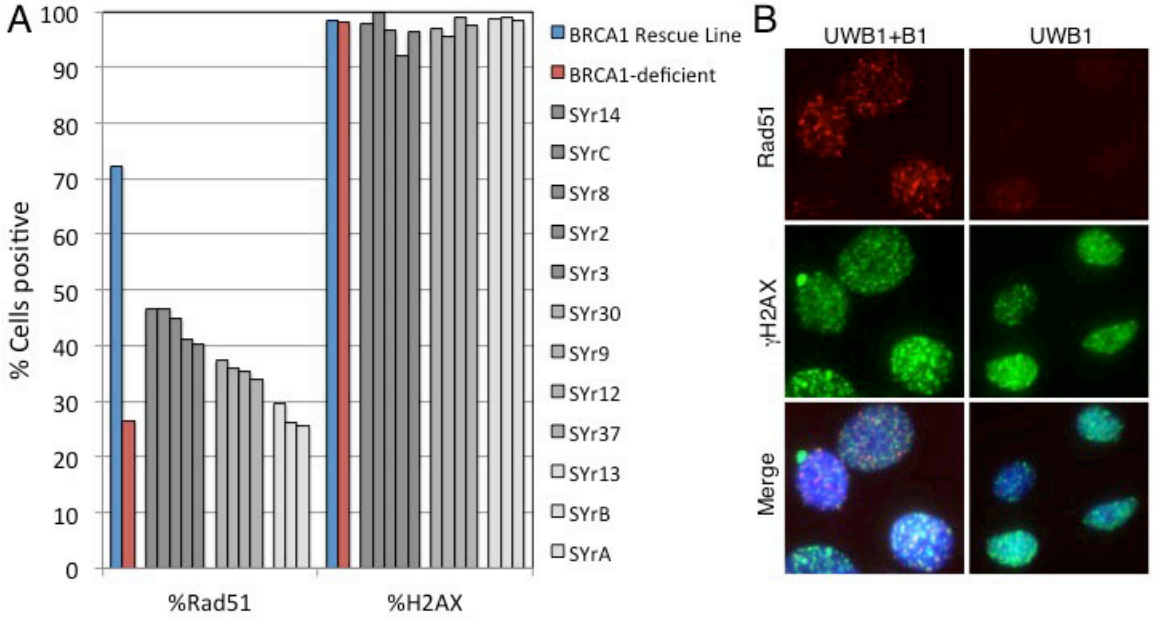

Figure 2: Derived BRCA1-deficient, PARPi resistant cell lines retain some level of homologous recombination (HR) ability, even in the absence of BRCA1. (A) Quantification of Rad51 loading, a marker of HR ability, and γH2AX, a marker of DNA damage, following treatment of cells with 10Gy IR. Positive cells contain greater than eight foci. The BRCA1-rescue line loads Rad51 efficiently, while the BRCA1-deficient line exhibits a defect in Rad51 loading. The derived PARPi resistant lines have varying levels of HR ability, some of which have restored HR ability, while others have levels comparable to the parental cell line. All cells show a similar level of γH2AX, demonstrating cells all received similar levels of damage. (B) Representative images of the BRCA1 rescue cell line (UWB1+B1) or BRCA1-deficient cell line (UWB1) stained by immunofluorescence for γH2AX as a marker for DNA damage and Rad51 as a marker of HR.

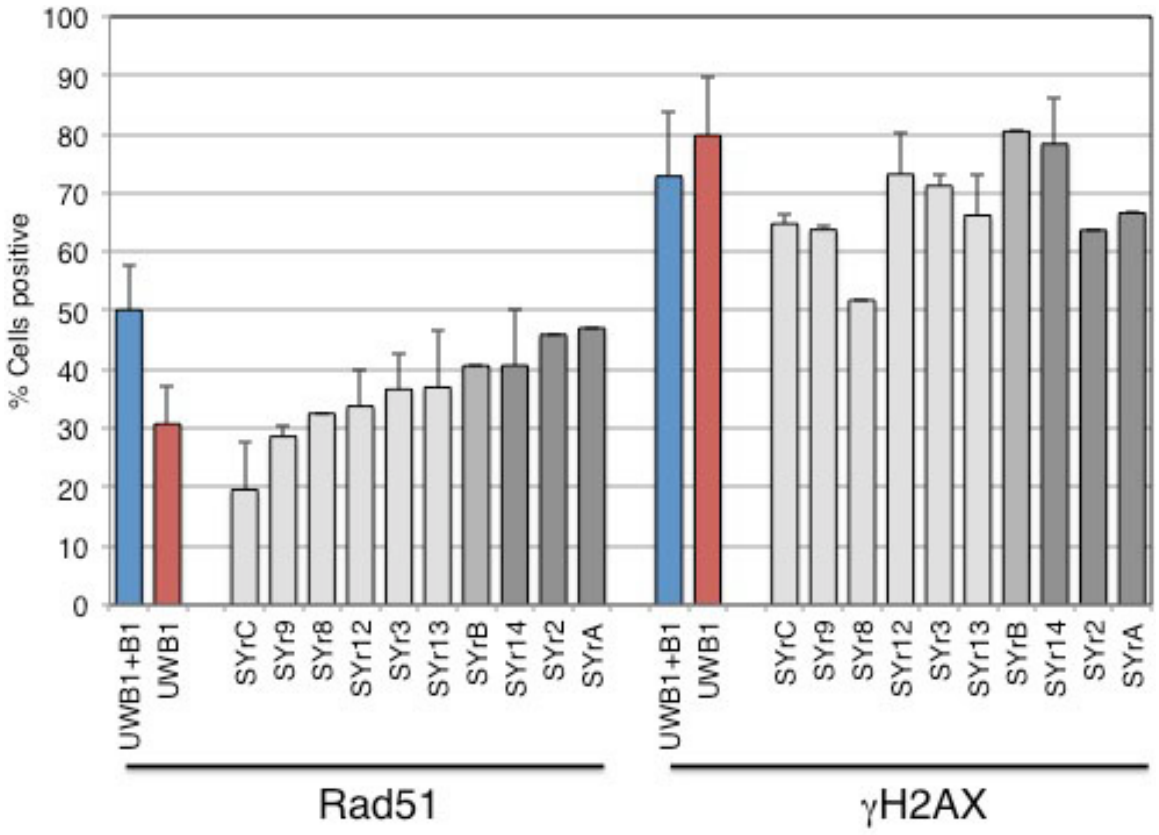

Figure 3: Derived BRCA1-deficient, PARPi resistant cell lines retain some level of homologous recombination (HR) ability, even in the absence of BRCA1. (A) Quantification of Rad51 loading, a marker of HR ability, and γH2AX, a marker of DNA damage, following treatment of cells with 10uM PARPi for 24hr. Positive cells contain greater than eight foci. The BRCA1-rescue line loads Rad51 efficiently, while the BRCA1-deficient line exhibits a defect in Rad51 loading, as when treated with 10Gy IR. The derived PARPi resistant lines have varying levels of HR ability, some of which have restored HR ability, while others have levels lower than the parental cell line. All cells show γH2AX staining, suggesting all were damaged by PARPi treatment. (B) Representative images of the BRCA1 rescue cell line (UWB1+B1) or BRCA1-deficient cell line (UWB1) stained by immunofluorescence for γH2AX as a marker for DNA damage and Rad51 as a marker of HR.

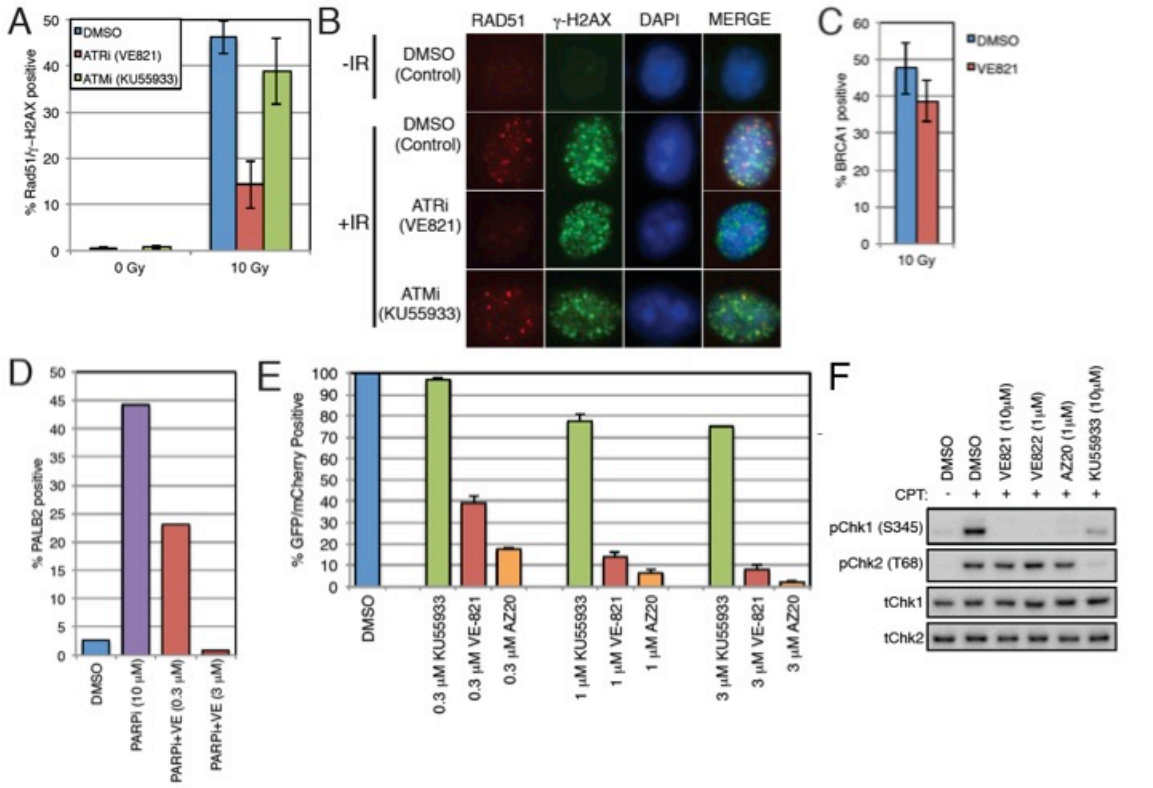

Figure 4: ATR inhibitor (ATRi) selectively inhibits homologous recombination downstream of BRCA1 but upstream of PALB2. (A)Quantification of Rad51 loading in HeLa cells, which retain BRCA1, 3 hours following 10 Gy IR treatment. Treatment with 10μM ATR inhibitor (VE821) significantly reduced Rad51 loading, while treatment with 10μM ATM inhibitor (ATMi) (KU55933) had little effect. (B) Representative images of HeLa cells in (A) stained for Rad51 and γH2AX staining. (C)Quantification of BRCA1 loading in HeLa cells 3 hours following 10 Gy IR treatment. Treatment with 10μM ATRi does not affect loading of BRCA1, indicating that ATRi prevents loading of Rad51 independently of BRCA1 loading. (D) Quantification of PALB2 foci in UWB1+B1 cells, which express wild-type BRCA1, following treatment with vehicle (DMSO), PARP inhibitor (AZD2281, 10 μM), or a combination of PARP inhibitor with ATR inhibitor at increasing doses (VE821, 0.3 μM or 3.0 μM). PARP inhibitor alone induces PALB2 loading as expected, while addition of ATR inhibitor prevents PALB2 loading in a dose dependent manner, indicating that ATR inhibitor prevents Rad51 loading by preventing the upstream component, PALB2, from being loaded. (E) FACS analysis of the DR-GFP reporter assay in U2OS cells following treatment of increasing doses of ATMi (KU55933) or two different ATRi's (VE821 or AZ20). Both ATRi's prevent homologous recombination, as indicated by the decreased percentage of GFP positive cells, while treatment with ATMi had little effect. (F) Western blot demonstrating that each inhibitor hits its known target following 1 hour 1 μM camptothecin treatment. All three ATRi's (VE821, VE822, and AZ20) inhibit ATR resulting in reduced pChk1 after the indicated dose, and the ATMi (KU55933) which inhibits ATM resulting in reduced pChk2.

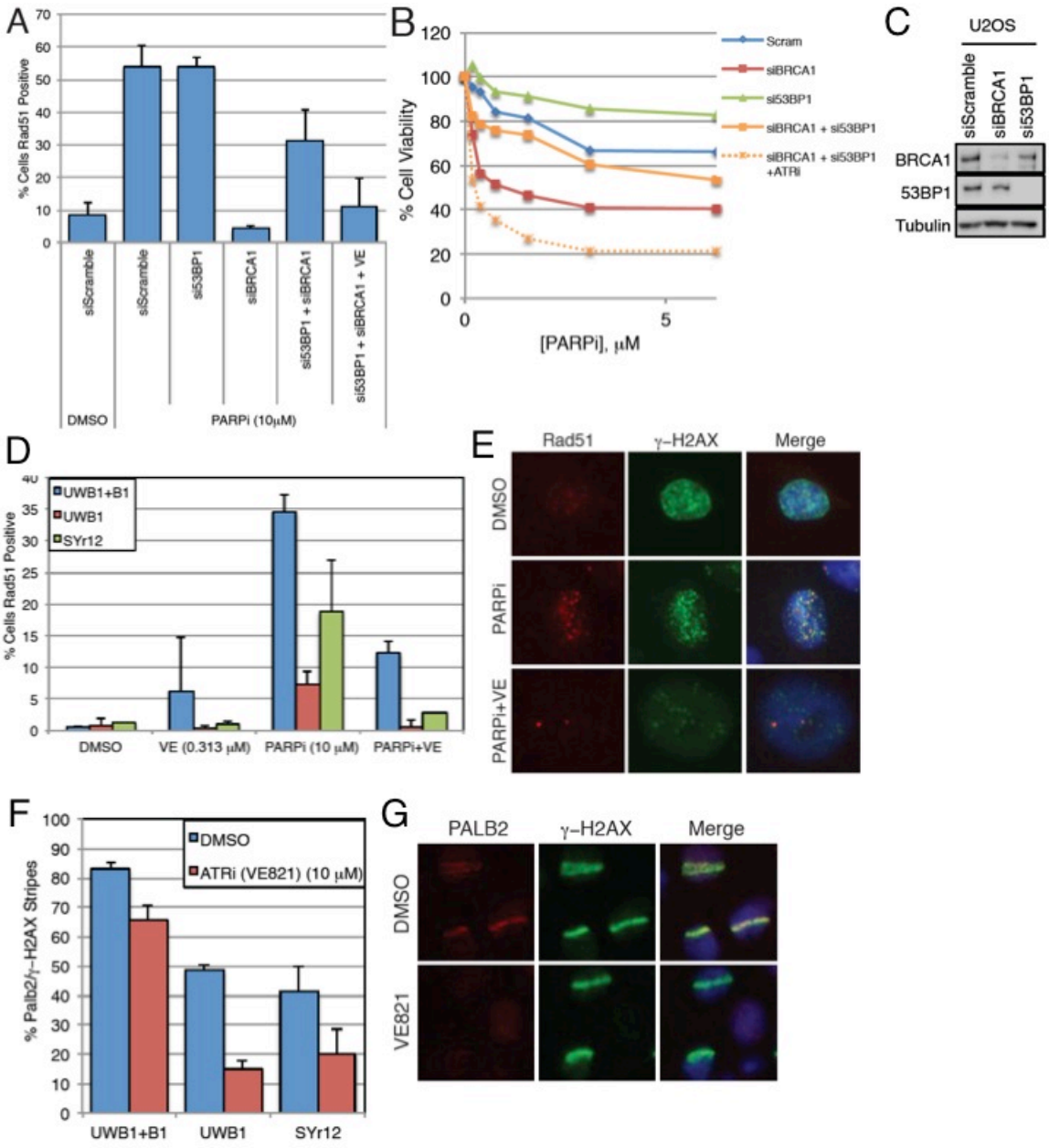

Figure 5: ATR inhibitor (ATRi) treatment results in decreased homologous recombination (HR) in BRCA1 deficient cells. (A) Quantification of Rad51 loading in U2OS cells after knock down of BRCA1, 53BP1, or the combination following treatment with PARPi or PARPi with ATRi. As expected, knockdown of BRCA1 results in decreased Rad51 loading, while knockdown of 53BP1 in combination rescues this defect. However, treatment with ATRi (0.3 μM) decreases this BRCA1-independent Rad51 loading. (B) Cell Titer Glo viability assay in U2OS cells following knockdown of BRCA1, 53BP1, or the combination following 5 days of treatment with the indicated dose of PARPi or PARPi with ATRi. As expected, following knockdown of BRCA1, cells are very sensitive to PARPi, but this sensitivity is rescued by co-depletion of 53BP1. However, cells are resensitized to PARPi by simultaneous treatment with ATRi. (C)

Western blot showing that the indicated siRNA depletes the expected target, either BRCA1 or 53BP1. (D) Quantification of Rad51 loading in UWB1+B1 rescue cells that express BRCA1, the BRCA1-deficient parental UWB1 cells, or the BRCA1-deficient, PARPi resistant SYr12 cell line following treatment with PARPi, ATRi, or the combination. All cells were able to load some level of Rad51 following PARPi treatment, though not as efficiently in the absence of BRCA1. Addition of ATRi (0.3 μM) resulted in decreased loading of Rad51 in all cell lines, although ATRi had the strongest effect on both of the BRCA1 deficient lines, almost completely eliminating all HR. (E) Representative images of Rad51 and γH2AX foci from (D). (F) Quantification of PALB2 loading at sites of UV laser stripe damage. Treatment with ATRi decreased the number of γH2AX stripes that were also PALB2 positive. Again, this effect was most pronounced in the BRCA1 deficient cells, suggesting ATR plays a critical role in loading of PALB2 in the absence of BRCA1. (G) Representative PALB2 and γH2AX laser stripes as described in (F).

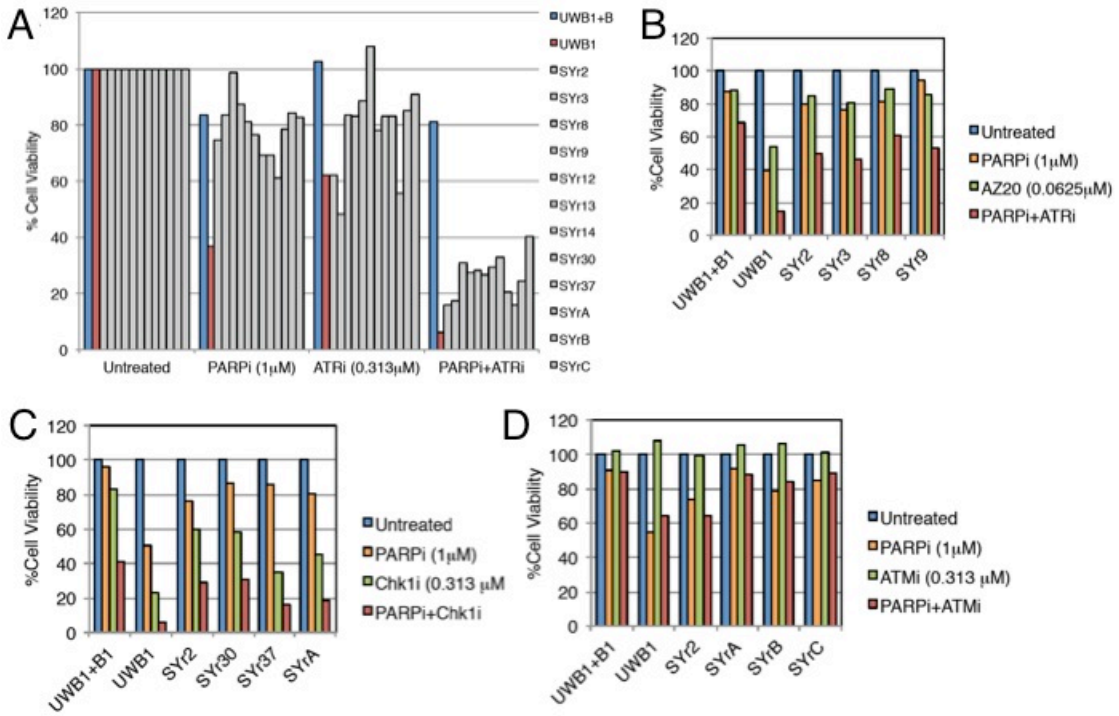

Figure 6: Combination of PARPi and ATRi results in decreased viability specifically in BRCA1-deficient cells. (A) Cell Titer Glo viability assay of BRCA1-rescue line UWB1+B1, BRCA1-deficient parental line UWB1, and the BRCA1-deficient, PARPi resistant lines (SYr cells) following treatment with DMSO, PARPi (1 μM), ATRi (VE821) (0.3 μM), or a combination of PARPi and ATRi. (B) Viability assay using the same cell lines as in (A) with a second ATRi, AZ20. (C) Viability assay using the same cell lines as in (A) with a Chk1i, which inhibits Chk1, a downstream effector of ATR. (D) Viability assay using the same cell lines as in (A) with an ATMi. The combination of PARPi and ATRi, either VE821 or AZ20, or the combination of PARPi and Chk1i, led to decreased viability specifically in the BRCA1-deficient cell lines, including the PARPi resistant cells. The combination of PARPi and ATMi did not have the same effect, suggesting this effect is specific to inhibition of the ATR pathway.

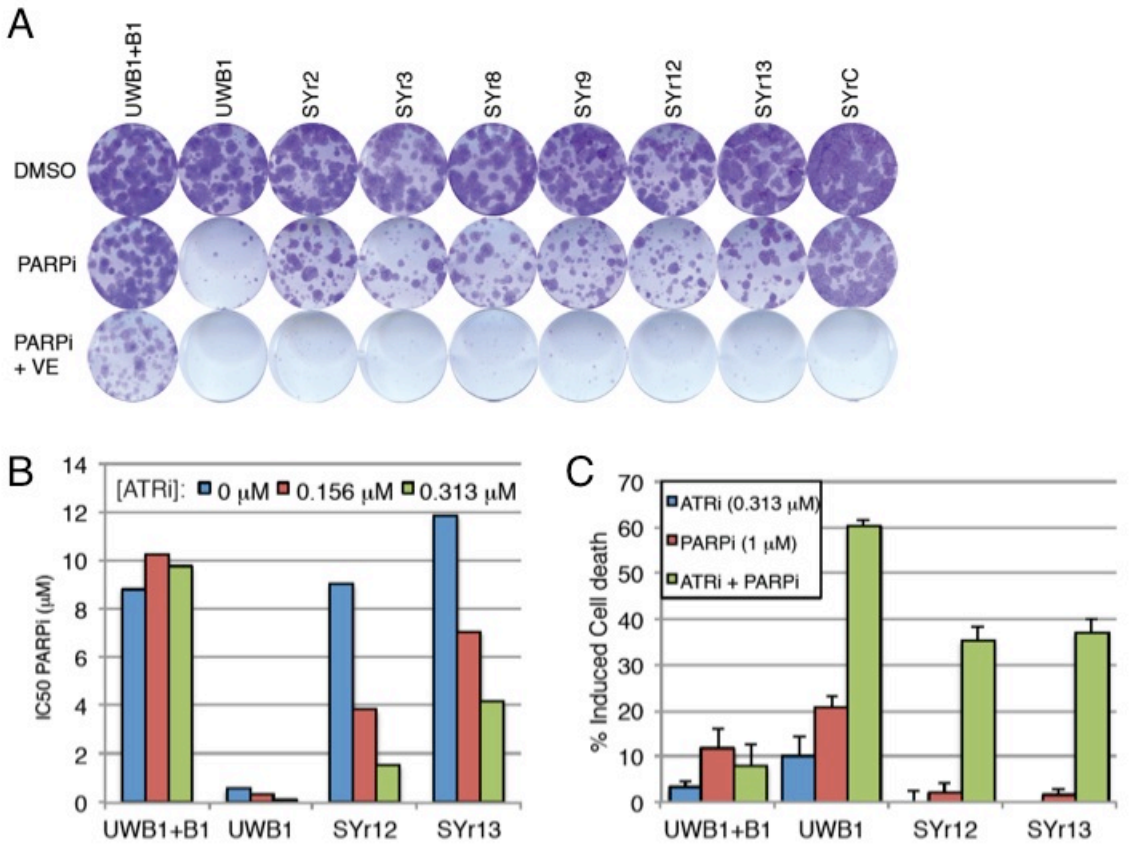

Figure 7: ATRi re-sensitizes PARPi resistant cell lines to PARPi and the combination of PARPi and ATRi results in cell death in BRCA1-deficient cells. (A) Crystal violet staining of colonies after 14 days of treatment with DMSO, PARPi (1 μM), or PARPi with ATRi (0.3 μM). While only the parental BRCA1-deficient cell line is sensitive to PARPi alone, all BRCA1-deficient cell lines, including the PARPi-resistant cells, are sensitive to the combination of PARPi and ATRi. (B) IC50 of each cell line, including the BRCA1-rescue UWB1+B1 cell line, the BRCA1-deficient parental UWB1cell line, and the PARPi-resistant cell lines, SYr12 and SYr13. Increasing doses of ATRi results in a decrease of the IC50 of SYr12 and SYr13 to PARPi, suggestion ATRi re-sensitizes these PARPi-resistant cells to PARPi in a dose dependent manner. (C) Quantification of AnnexinV staining by FACS analysis in the BRCA1-rescue UWB1+B1 cell line, the BRCA1-deficient parental UWB1cell line, and the PARPi-resistant cell lines, SYr12 and SYr13, following ATRi treatment, PARPi treatment, or the combination for 6 days. While only UWB1 cells undergo cell death after PARPi treatment alone, all BRCA1-deficient cells undergo apoptosis following treatment with the combination of PARPi and ATRi.

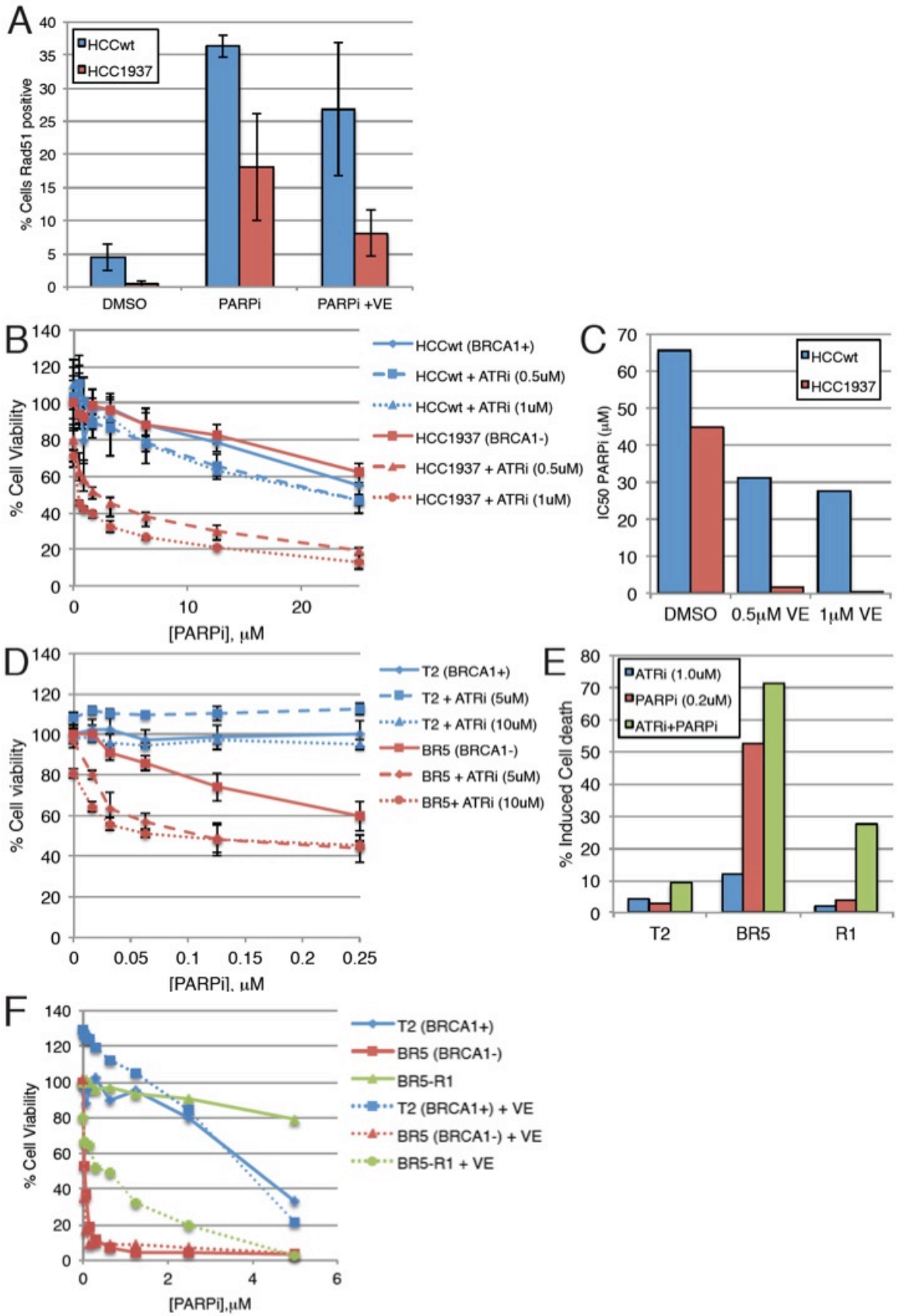
A
HCCwt
HCC1937
% Cells Rad51 positive
DMSO
PARPi
PARPi +VE
B
HCCwt (BRCA1+)
HCCwt + ATRi (0.5uM)
HCCwt + ATRi (1uM)
HCC1937 (BRCA1-)
HCC1937 + ATRi (0.5uM)
HCC1937 + ATRi (1uM)
% Cell Viability
[PARPi], μM
C
HCCwt
HCC1937
IC50 PARPi (μM)
DMSO
0.5μM VE
1μM VE
D
T2 (BRCA1+)
T2 + ATRi (5uM)
T2 + ATRi (10uM)
BR5 (BRCA1-)
BR5 + ATRi (5uM)
BR5+ ATRi (10uM)
% Cell viability
[PARPi], μM
E
ATRi (1.0uM)
PARPi (0.2uM)
ATRi+PARPi
% Induced Cell death
T2
BR5
R1
F
T2 (BRCA1+)
BR5 (BRCA1-)
BR5-R1
T2 (BRCA1+) + VE
BR5 (BRCA1-) + VE
BR5-R1 + VE
% Cell Viability
[PARPi],μM

Figure 8: Decreased cell viability in multiple BRCA1-deficient cell lines following treatment with the combination of PARPi and ATRi. (A) Quantification of Rad51 foci following PARPi treatment in human breast cancer, BRCA1-deficient HCC1937 cells or HCCwt which exogenously express BRCA1. As expected, BRCA1-deficient HCC1937 cells have a partial defect in Rad51 loading following PARPi treatment; however, HCC1937 cells have an even greater defect in Rad51 loading following the combination of PARPi and ATRi treatment. (B) Cell Titer Glo viability assay of BRCA1-deficient HCC1937 cells and HCCwt cells which exogenously express BRCA1, following treatment with PARPi in the presence or absence of ATRi. HCC1937 cells, which are resistant to PARPi treatment, are re-sensitized to PARPi following addition of ATRi. (C) IC50 of each cell line, including the BRCA1-rescue HCCwt cell line and the BRCA1-deficient HCC1937. Increasing doses of ATRi results in a decrease of the IC50 of HCC1937 to PARPi, suggestion ATRi re-sensitizes this PARPi-resistant cell line to PARPi in a dose dependent manner. (D) Cell Titer Glo viability assay of the mouse ovarian cancer, BRCA1-deficient BR5 cells and the mouse ovarian cancer T2 cell line which expresses wtBRCA1, following treatment with PARPi in the presence or absence of ATRi. BR5 cells, which are sensitive to PARPi treatment, are even more sensitive to PARPi following addition of ATRi. (E) Quantification of AnnexinV staining by FACS analysis in the BRCA1-expressing T2 cell line, the BRCA1-deficient parental BR5 cell line, and the PARPi-resistant cell line, R1, following ATRi treatment, PARPi treatment, or the combination for 6 days. While only BR5 cells undergo cell death after PARPi treatment alone, both BRCA1-deficient cells undergo apoptosis following treatment with the combination of PARPi and ATRi. (F) Cell Titer Glo viability assay of the mouse ovarian cancer, BRCA1-deficient BR5 cells, the mouse ovarian cancer T2 cell line which expresses wtBRCA1, and the derived PARPi resistant line, R1, following treatment with PARPi in the presence or absence of ATRi. R1 cells, which are resistant to PARPi treatment, are re-sensitized to PARPi following addition of ATRi.

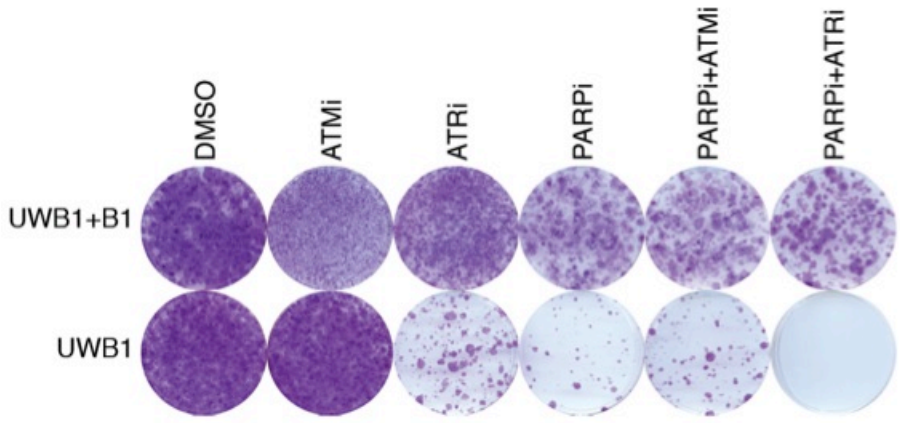

Figure 9: The combination of PARPi and ATRi prevent resistant colonies from emerging in a BRCA1-deficient cell line. Crystal violet staining of colonies and emerging following 45 days of treatment with ATMi (0.3 μM), ATRi (0.3 μM), PARPi (1μM) or the combinations of PARPi and ATMi or PARPi and ATRi. While there was no sensitivity to any of the single agents or combination in the BRCA1-rescue UWB1+B1 cell line, the BRCA1-deficient UWB1 cells were sensitive to ATRi and PARPi. While most UWB1 cells died following PARPi treatment, several visible, resistant colonies emerged after 45 days. However, no resistant colonies emerged when ATRi was included with the PARPi treatment. This suggests that ATR is necessary for PARPi resistance to emerge, and the combination of ATRi and PARPi prevents PARPi resistance.